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Biological, Serological and Molecular Characterization of a *Potato Y Potyvirus* Strain in Egypt

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ABSTRACT

A *Potato virus Y potyvirus* (PVY) strain was isolated from naturally infected potato plants showing mild mosaic and malformation. Isolation performed depending on indirect enzyme-linked immunosorbant assay (I-ELISA) using monoclonal antibodies, and by mechanical inoculation on *Chenopodium amaranticolor* as local lesions host. Tobacco cultivars White Burley, Turkish, Samsun, *N. rustica* and *N. glutinosa*, *Lycopersicon esculentum* cv. Duke, *Solanum tuberosum* cv. Alpha, *Datura metel* and *Petunia axillaris* revealed systemic mild mosaic and malformation. Local symptoms in the form of chlorotic local lesions observed on *Ch. amaranticolor* and *Ch. quinoa*, while no symptoms appeared on *D. stramonium*. *Myzus persicae* was able to transmit PVY in a non-persistent manner. Light microscopic examination revealed amorphous inclusion bodies in epidermal strips cells of PVY infected tobacco. Electron microscopy of the same infected tobacco tissue showed pinwheels, scrolls and laminated aggregates. Purified virus concentration calculated to be 3.1 mg/ml, also preparation appeared with good purity, as A_{260}/A_{280} ratio was 1.51. Electron micrograph of PVY purified preparation showed flexuous filaments particles with model length of 730×13 nm. A specific antiserum produced, IgGs purified and its titer was determined using I-ELISA, which reacted up to dilution of 1/1024 with clarified infectious sap. Positive results in the form of precipitation zones observed in Ouchterlony agar and the simple single diffusion test in agar gave precipitations surrounding the infected tobacco tissues. Tissue and dot blot immunoassays (TBIA & DBIA) used for IgGs evaluation. TBIA gave positive results with the infected tobacco tissues. Concerning DBIA, positive results were obtained with the purified virus preparations and with the infectious sap dilutions till 10^{-6}. Sodium dodecyl sulfate-polyacryalamide gel electrophoresis (SDS-PAGE) show that the molecular weights of PVY capsid protein was approximately 34 KDa. The agarose gel of the multiplex immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR) for differentiation of PVY strain using six specific primers pairs indicated two expected bands sizes with the understudy isolate similar to PVY^O control strain.

KEYWORDS: *Potato Y Potyvirus*, Isolation, Symptomatology, Microscopy, Purification, Antiserum production& Evaluation, SDS-PAGE, Multiplex IC-RT-PCR.

INTRODUCTION

The viruses of the *Potyviridae* infect wide range of economical crops **(Edwardson, 1974)**, and the representative virus for such family is *potato Y potyvirus* (PVY) which infects potato, the most important crop in the developing countries **(Hollings et al., 1976 and Sabir, 2012)**. In Egypt, PVY was isolated from potato plants by several workers **(Gamal El-Din et al., 1997; El-Absawy et al., 2012)**.

PVY strains were distinguished according to their symptoms produced on *Nicotiana tabacum* cv. White Burley, i.e., PVY^O (common strain) induces mosaic and mild malformation, PVY^C (streak strain) induces mosaic and vein banding, PVY^N (necrotic strain) produces systemic necrotic regions **(Brunt et al., 1996; Boonham et al., 2002; Karasev and Gray, 2013)**.

PVY^{NTN} strain considered a subgroup of the PVY^N strain group, which appears to be different to other isolates reported that produces vein necrosis on tobacco necrotic rings on potato tubers but that does not react with monoclonal antibodies specific to PVY^N **(Gamal El-Din et al., 1997; Amer et al., 2004; Ramírez-Rodríguez et al., 2009)**.

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The PVY genome is a single stranded positive sense RNA approximately 10 Kb in length, with a genome link protein (VPg) at the 5-terminus, and a poly A single at the 3-terminus, encapsidated by about 2000 copies of a single coat protein of approximately 34 kD size **(Dougherty and Hiebert, 1980; Quintero-Ferrer Karasev, 2013)**.

The genomes of potyviruses consisting of genes that encoding a different viral proteins; N terminal protein (P1), helper component protease (HC-Pro), protein P3, 6KD protein (6K1), cytoplasmic inclusion protein (CIP), 6KD protein 2 (6K2), genome-linked protein (VPg), nuclear inclusion protein A (NIa), nuclear inclusion protein B (NIb) and coat protein (CP) **(Riechmann et al., 1992; Shand et al., 2009)**.

Several molecular methods developed to distinguish between PVY isolates and PVY^{NTN} isolates. One is based around sequence differences at the 5' Terminal end of the genome **(Rosner and Maslenin, 1999)**, one at the 3' end of the genome **(Rosner and Maslenin, 2001)**, one including exploitation of the coat protein recombination event **(Boonham et al., 2002)**, whilst other are based on variability across the entire genome **(Glais et al., 1998)**. A multiplex RT-PCR assay developed by **Nie and Singh (2002)** queries the P1 cistron and differentiates PVY into two main groups: those that produce leaf necrosis on tobacco indicator plants (European PVY^{N}, European PVY^{NTN}, $PVY^{N:O}$ (or PVY^{N-Wi}), North American PVY^{N} and North American PVY^{NTN}) and those that do not produce leaf necrosis on tobacco (PVY^{O}) **(Nie and Singh, 2003)**. The **Nie and Singh (2002)** assay can detect mixtures of PVY^{O} plus PVY^{N} (including PVY^{NTN} and $PVY^{N:O}$). A second multiplex assay, developed by **(Nie and Singh, 2003)**, was used in conjunction with the P1 assay to identify isolates capable of producing necrotic lesions on tobacco leaves, and it also identifies recombinants of the PVY^{NTN} and $PVY^{N:O}$ types based on amplification of three amplicons or one amplicon, around the respective recombination junctions. A third assay is based on *Hinc*II restriction digests of P1 amplicons and can differentiate PVY^{O} from the necrotic strains of PVY^{N}, PVY^{NTN}, $PVY^{N:O}$, PVY^{NA-N}, and PVY^{NA-NTN} **(Crosslin et al., 2005)**. This assay will also detect strain mixtures of PVY^{O} and PVY^{N}, PVY^{NTN}, NA-PVY^{N}, or NAPVY^{NTN}.

There are four types of cytoplasmic cylindrical inclusions induced by the members of *Potyviridae,* which appeared under electron microscope in the form of pinwheels and laminated aggregates, while those inclusion aggregates appeared under light microscope as irregular granular amorphous aggregates **(Edwardson et al., 1984)**. These types of inclusions are: Type I: pinwheels and scrolls, Type II: pinwheels and laminated aggregates, Type III: pinwheels, scrolls and laminated aggregates and Type IV: pinwheels, scrolls and short curved laminated aggregates **(Boonham et al., 2002)**.

PVY has flexuous filamentous particles with model length of 730×11 nm **(Delgado-Sanchez and Grogan, 1966; Brunt, 2001)**.

Makkouk and Gumpf (1976) found that using a scheme depending on the combination between the intravenous and intramuscular injections was more efficient for producing PVY specific polyclonal antisera with high titer.

Different agar gel diffusion tests in plates used for detection and identification of the elongated viruses in plant extracts and for determination of virus dilution ends in both purified preparations and infectious sap **(Ouchterlony, 1962; Slack and Shepherd, 1975; Gumedzoe, 1993 and Bodaghi et al., 2000)**.

Tissue blotting immunoassay (TBIA) is an easy and simple technique for detection of plant viruses in plant tissues directly as the sample preparation is reduced to the minimum **(Yu et al., 2002 and Asins et al., 2004)**. Dot blot immunobinding assay (DBIA) found to be cheaper and more sensitive compared with ELISA because the protein binding capacity of the nitrocellulose membrane is higher than polystyrene of the ELISA plates **(Dijkstra and De Jager, 1998)**.

The aim of this investigation is to isolate PVY strain from potato plants, and identifying it depending on some biological, serological and molecular characteristics.

MATERIALS AND METHODS

Virus isolation, serological identification and symptomatology

Potato (*Solanum tuberosum*cv. Spunta) plants showing mild mosaic and malformation were collected from the open fields of Mansoura, El-Dakahlia Governorate, Egypt. Samples were then tested by indirect enzyme linked immunosorbent assay (I-ELISA) using specific monoclonal antibodies (mAbs) against PVY^{O}, PVY^{C} and PVY^{N} strains as demonstrated by **Koenig (1981)**. For such purpose the following antibodies were used: 1) PVY^{O} and PVY^{C} specific mAbs (SASA, UK), 3) PVY^{N} specific mAbs (Bioreba 112712). Isolation was performed depending on I-ELISA and single local lesions produced on

Chenopodium amaranticolor. Virus was maintained in tobacco (*Nicotiana tabacum* cv. White Burley) plants by mechanical sap inoculation within an aphid-proof cage under normal glasshouse conditions.

Sap extracted from PVY infected tobacco using 0.05 M phosphate buffer (pH 7.2) (1:1 w:v) was used for the mechanical inoculation of differential hosts using carborandom as an abrasive. Differential hosts used were: *Nicotiana tabacum* cvs. White Burley, Turkish and Samsun, *N. glutinosa*, *N. rustica*, *Lycopersicon esculentum* cv. Duke, *Solanum tuberosum* cv. Alpha, *Datura metel*, *D. stramonium* and *Petunia axillaris* (Solanaceae), *Ch. amaranticolor*, *Ch. quinoa* (Chenopodiaceae) and *Gomphrena globosa* (Amarnthiaceae).

Insect transmission

To obtain virus-free aphids, a culture of *Myzus persicae* was started from a non-viruliferous single virginiparous female reared on healthy cabbage plant (obtained from Plant Protection Research Institute, ARC, Dokki, Giza Governorate, Egypt). Aphis kept in Petri dish for one hour for starvation, and transferred to feed for 30 min acquisition feeding period on healthy and PVY infected *N. tabacum* cv. White Burley. Aphids were then allowed to feed for 1 h inoculation feeding period on *N. tabacum* cv. White Burley healthy seedlings (carrying 5 leaves) kept in an insect-proof cages (10 aphids per plant and 5 plant for each feeding source). Plants sprayed with insecticide then assayed for virus infection using I-ELISA 15 days later.

Light and electron microscopy

Inclusions bodies detected in epidermal strips obtained from PVY infected tobacco leaves (20 days post inoculation). Amorphous inclusions were observed with light microscope after staining strips using a mixture of 0.5% methyl green and pyronine Y (MGP-Y) **(Christie and Edwardson, 1977)**.

For the electron microscopic examination a method for samples preparation was performed as described by **Luft (1961)** with modifications: Infected leaves (30 days post inoculation) were cut to 1×1 mm pieces. Pieces were fixed for 4 h at 4 ºC in 0.08 M cacodylate buffer pH 7.4 containing 5 % glutaraldehyde and 4 % paraformaldehyde. The fixed specimens were washed three times at half hour intervals with 0.1 M cacodylate buffer pH 7.4, containing 3 % sucrose. These samples were post fixed for 40 min at 4 ºC in 1 % osmium tetroxide dissolved in a solution of 0.1 M cacodylate buffer pH 7.4 and 2 % sucrose. Following three hours wash in 0.1 M cacodylate, 3 % sucrose pH 7.4, samples were dehydrated in ascending concentration of alcohol series, sequentially followed by propylene oxide, then propylene oxide plus Epoxy resin (1:1 v:v) and finally embedded in pure Epoxy resin. Thin sections were cut, then stained with uranyl acetate and lead citrate and viewed with a Philips 400T transmission electron microscope (Electron Microscope Unit, Ain Shams Specialized Hospital, Cairo, Egypt).

Virus purification

Two hundred grams of systematically PVY infected leaves from *N. tabacum* cv. White Burley were frozen in liquid nitrogen and ground into a fine powder. The virus was purified using differential ultracentrifugation as mentioned by **Abdel Salam et al., (1989)**. The final pellets were resuspended in 1 ml of 0.01 M Tris-HCl buffer (pH 7.2), containing 0.2 % 2-Mercaptoethanol, 0.005 M citric acid, 0.5 M urea, 0.2 % Triton X-100 (v/v) and kept at 4 °C.

The purified preparation was negatively stained as described by **Milne and Lesemann (1984)** and the grids were examined with the previously mentioned electron microscope. Purified preparation was also evaluated via spectrophotometry and viral yield was calculated according to the equation given by **Noordam (1973)**.

Antiserum production

Antiserum against the PVY was prepared by injecting two adult New Zealand white rabbits (2-4 kg) with purified viral preparation according to the method described by **Makkouk and Gumpf (1976)**. The injections were at one week interval, first dosage (3 mg) was taken intramuscularly with Freund's complete adjuvant, the second one was injected intravenously (1 mg). The third and the fourth injections (3 mg each) were taken intramuscularly with incomplete adjuvant. Prior to applying the injection schedule normal serum was obtained by bleeding the rabbits. After removing the blood clot, clarified sera were stored at -20 °C.

Purification and titration of immunoglobulins G (IgG)

IgGs were isolated from the antiserum according to **Steinbuch and Audran (1969)**. One ml of virus antiserum was added to 2 ml of 0.06 M sodium acetate buffer (pH 4.8) and dialyzed three times

against the same buffer, 0.082 ml Caprylic acid was added with stirring and left for 30 min at 28 ˚C. Mixture was centrifuged at 8000 rpm for 10 min and supernatant was dialyzed two times against 0.05 M phosphate buffer (pH 7.2) for 4 h. The IgGs were precipitated using saturated ammonium sulfate and collected by centrifugation at 8000 rpm for 10 min. Pellets were resuspended in 1 ml distilled water. Concentration of IgG was adjusted to 1 mg/ml (1.4 OD at 280 nm) and stored at -20 ˚C.

IgGs were diluted with ELISA coating buffer two fold dilutions (from 1:2 up to 1:4096). Dilution end points (DEP) were determined with I-ELISA using clarified infected tobacco leaves sap which was prepared by extraction in sample buffer at a ratio of 1:10 (w/v), while healthy tobacco clarified sap was used as a control.

Evaluation of purified IgGs in detecting PVY

a) Diffusion tests in agar

Double diffusion test in agar (Ouchterlony) was carried out according to the method of **Purcifull and Batchelor (1977)** with some modifications. Agar (Sigma Chemical Co., USA) was prepared (1 %) in 0.05 M phosphate buffer (pH 7.2) containing 0.1 % sodium azide and 0.2 % sodium dodecyl sulfate (SDS). Agar was fully melted and allowed to cool to 50 °C, then poured in plastic Petri dishes to depth of 3 mm. After solidification, a central and 6 peripheral wells were cut out using patterned template. Central well was filled with ½ dilution of the purified IgGs while peripheral wells were filled with tobacco infectious clarified sap (extracted in 0.05 M phosphate buffer containing 0.5 % SDS (pH 7.2) at rate of 1:1 (w/v) and boiled for 5 min in a water bath for virus disruption). Results were observed 24-48 h post incubation in a humid box at 37 °C.

Another Simple single diffusion test in plates was performed according to **Slack and Shepherd (1975)**. Agar (2 %) was prepared in 0.05 M Tris buffer (pH 7.2) containing 0.1 % sodium azide, 0.85 % NaCl and 0.2 % SDS. Agar was fully melt, then allowed to cool to 45 °C, antiserum (dilution ½) was added to agar with a rate of 1:1 (v:v), and was mixed gently then poured into the plastic Petri dishes to a depth of 3 mm and left to set. Samples (small pieces of about 3×3 mm) from infected, healthy tobacco leaves were slightly embedded on agar surface after performing slight pressure on the tissue using forceps. Plates were incubated at room temperature (30 °C) for 20 h in moisturized atmosphere, and the results were observed as zones of precipitation in agar.

b) Tissue and dot blot immunoassays (TBIA& DBIA)

TBIA was carried out as given by **Lin et al. (1990)** with modifications as follows: infected and healthy leaves were cut to parts (0.5×0.5 cm), then stamped on a nitrocellulose membrane (0.45 µm) (Millipore Corp., USA) after wetting the membrane by immersing in phosphate buffer saline tween (PBST) for few seconds to remove air and left to dry. Membrane was left for 20 min for sap to be adsorbed, plant tissues were then removed. The Membrane was then placed in blocking solution (2 % bovine serum albumin in phosphate buffer saline (PBS)) on a shaker for 30 min at room temperature, then incubated with primary antibodies (½ dilution of the purified IgGs) for 1 h at 37 °C on a shaker. After washing 3 times, 10 min each with PBST, the membrane was incubated with conjugated anti-IgGs (dilution 1/5000) for 1 h at 37 °C on a shaker and washed as mentioned.

The substrate solution consists of 14 mg of nitroblue tetrazolium (NBT) and 7 mg 5-bromo-4-chloro-3-indolylphosphate (BCIP) dissolved in 40 ml of substrate buffer (0.1M Tris-HCl (pH 9.6), 0.1M NaCl and 5 mM $MgCl_2$) was prepared. Thereafter the membrane was incubated in a volume of 20 ml of such solution for 10-20 min under dark conditions. When purple color developed clearly reaction was stopped by placing membrane in Tris-EDTA (TE) buffer (10 mM Tris-HCl (pH 8.0) and 1mM EDTA).

Concerning DBIA, depending on **Hibi and Saito (1985)** method, Infected clarified tobacco sap was diluted to 10 fold dilutions using PBS, also purified virus preparations were diluted to 10^{-1} using PBS and healthy undiluted tobacco sap were used as a controls. Nitrocellulose membrane was placed for few seconds in PBS to remove air and left to dry inside a clean Petri dish. Samples were dropped (5 µl each) on the membrane and were left to dry. Membrane was then placed in blocking solution as mentioned and the rest of the procedure was carried out as previously described.

SDS-PAGE of viral coat protein

The molecular weight of PVY coat protein subunits was determined by sodium dodecyl sulfate-polyacryalamide gel electrophoresis (SDS-PAGE) using 4 % stacking gel on a 12 % resolving gel and a buffer system as described by **Laemmli (1970)**.

Multiplex IC-RT-PCR for differentiation of PVY strain

The immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR) performed using infected tobacco leaf collected 15 days post PVY understudy strain, PVY^O and PVY^N strains (controls identified during previous work using specific mAbs) inoculation. Immunocapturing and cDNA synthesis carried out as described by **Minafera and Hadidi (1994)**:

The wells of an ELISA plate were coated by adding 200 µl of IgGs (diluted to 10-1 using coating carbonate buffer (pH 9.6)) and incubated 4 h at 37 °C, after that, plate was washed 3 times with PBST, 5 min each. Plant tissues were ground in sample buffer (1 gm : 5 ml), then centrifuged at 3000 rpm/10 min at 4 °C, 200 µl of the supernatant were added to each pre-coated wells and incubated overnight at 4 °C, then plate was washed 3 times, 5 min each with PBST. Twenty five of pre-heated transfer buffer (10 mM Tris-HCl (pH 8.0) containing 1 % Triton X-100) were added to each well and kept at 65 °C for 5 min. Aliquots of 5 µl from the resulting released RNA solutions were immediately transferred to microcentrifuge tube. To each tube the following components added and incubated at 42 °C/2 h for cDNA synthesis: 1 µg of oligo-dT primer, 4 µl of 5X first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl , 15 mM $MgCl_2$), 3 µl of 0.1 M dith-iothreitol (DTT), 5 µl of 0.3 M 2-ME, 2.5 µl of 10 mM of each deoxynucleotide triphosphate (dNTPs), 1 µl RNasin (Promega Corp, USA) (40 unit) and 1 µl of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (200 unit). Final product used immediately for PCR reaction.

Multiplex PCR was performed and primers were designed as described by **Lorenzen et al. (2006) (Table 1 & 2)**, in a 20-µl reaction volume that contained 0.8 µl from the produced cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.8 mM $MgCl_2$, 0.2 mM from each dNTP, 0.12 µM from each of six primer pairs (Invitrogen Corp., USA), and 1.0 unit Taq DNA polymerase (Promega Corp, USA). The PCR performed using PerkinElmer Cetus Thermal Cycler PE 9700 (PerkinElmer Inc., USA) with a program consisted of denaturing at 94 °C for 2 min, 12 cycles of 94 °C for 10 s, 66 °C for 30 s (minus 0.5 °C per cycle), and 60 s at 72 °C, followed by 20 cycles of 92 °C for 10 s, 60 °C for 30 s, and 72 °C for 60 s, ending with a final extension for 7 min at 72 °C.

For PCR product analysis, 1.5 % agarose gel used and electrophoresis carried out in Sub-Cell DNA apparatus (Bio-Rad® Lab., USA) at 80 V. The amplified gene band visualized on an UV Transilluminator and photographed by Gel Documentation System (AlphaImager® TM1220, Documentation and Analysis system, Canada).

Table (1): PVY primers used in the multiplex PCR assay and their genomic locations

Primer*	Polarity	Sequence (5′-3′)	Start Position
o2172a	+	CAACTATGATGGATTTGGCGACC	2172
n2258	+	GTCGATCACGAAACGCAGACAT	2258
o2439c	-	CCCAAGTTCAGGGCATGCAT	2487
n2650c	-	TGATCCACAACTTCACCGCTAACT	2650
n5707	+	GTGTCTCACCAGGGCAAGAAC	5707
o6266c	-	CTCCTGTGCTGGTATGTCCT	6266
S5585m	+	GGATCTCAAGTTGAAGGGGAC	5584
A6032m	-	CTTGCGGACATCACTAAAGCG	6033

* Primers named depending on start position and strain name according to **Lorenzen et al. (2006)**.

Table (2): Expected sizes of PCR products and target strains for PVY primer combinations in the multiplex assay

Primer combination	Size (bp)	Target strain
n2258 + o2439c	181	NTN, N:O
o2172 + o2439c	267	O
o2172 + o2439c	398	N
n5707 + A6032m	328	N, NA-N/NTN
S5585m + A6032m	452	NTN
S5585m + o6266c	689	N:O, O

RESULTS

Virus isolation, serological identification and symptomatology

Potato virus Y potyvirus (PVY) was isolated from naturally infected potato plants showing mainly mosaic and malformation. Samples gave positive I-ELISA results with PVY^O and PVY^C specific mAbs

while negative results were obtained with PVY^N specific mAbs. Samples with positive results produced chlorotic local lesions on *Ch. amaranticolor* **(Figure 1, A)**. Local lesion isolation was performed and the produced lesions were inoculated on *Nicotiana tabacum* cv. White Burley plants.

Obtained data showed that the isolated PVY strain gave mild mosaic and vein clearing which convert to small necrotic lesions on tobacco cultivar White Burley **(Figure 1, B& C).** Tobacco cultivars Turkish, Samsun, *N. rustica* and *N. glutinosa*, *Lycopersicon esculentum* cv. Duke **(Figure 1, D)**, *Solanum tuberosum* cv. Alpha, *Datura metel* **(Figure 1, E)** and *Petunia axillaris***(Figure 1, F)** revealed systemic symptomsin the form of mild mosaic and malformation. Local symptoms in the form of chlorotic local lesions were observed on *Chenopodium amaranticolor* and *Ch. quinoa*, while no symptoms appeared on *D. stramonium*.

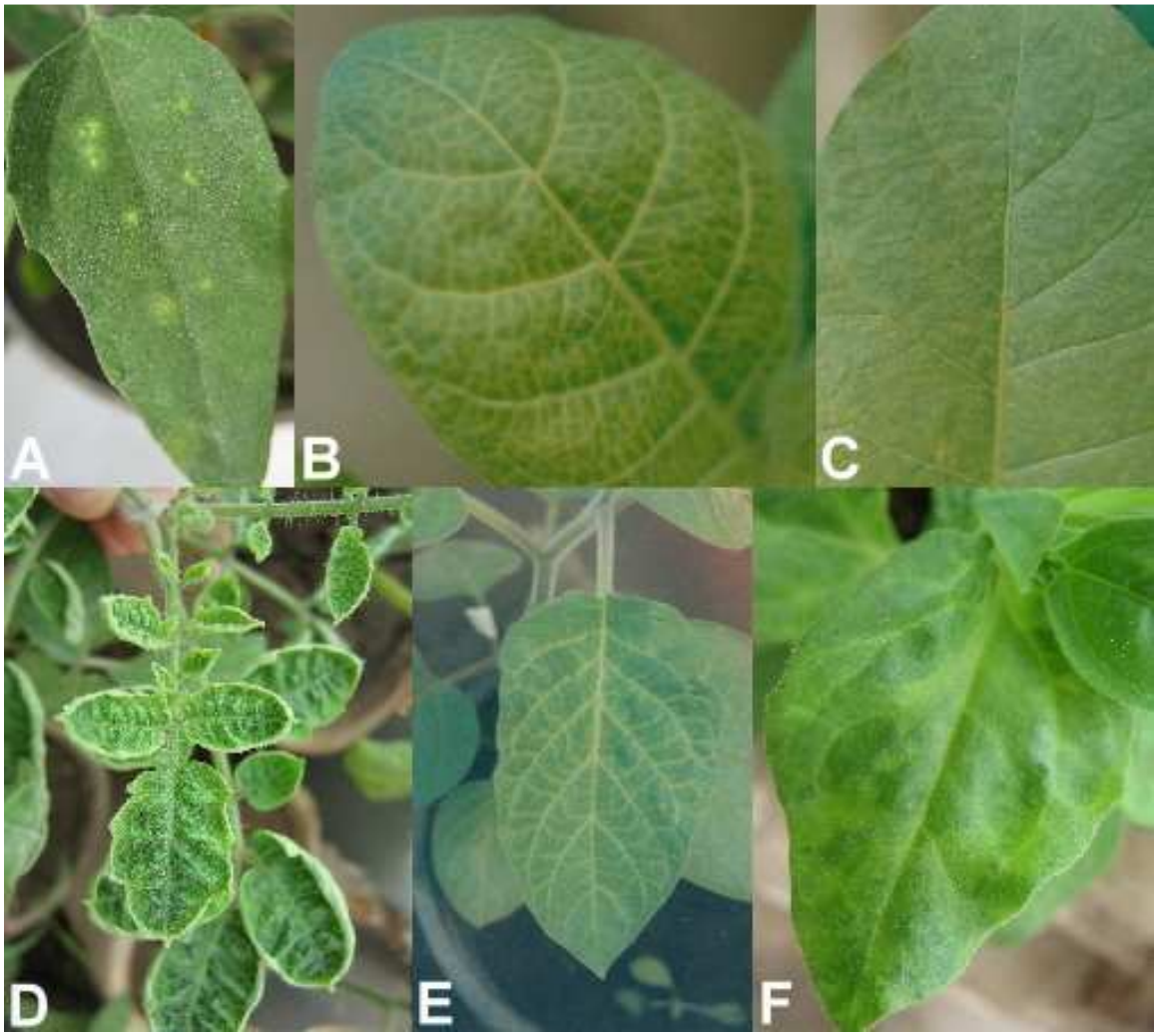

Figure (1): (A) Isolation of PVY depending on chlorotic local lesions produced on *Ch. amaranticolor*. Differential hosts mechanically inoculated with PVY isolate giving: mild mosaic and vein clearing (B) which convert to small necrotic lesions (C) on *N. tabacum* cv. White Burley. Mild mosaic and malformation were observed on *L. esculentum* cv. Duke, *D. metel* and *P. axillaris* (D, E & F, respectively).

Insect transmission

Results showed that *Myzus persicae* was able to transmit PVY in a non-persistant manner from infected to healthy *N. tabacum* cv. White Burley seedlings, since systemic symptoms and positive I-ELISA results were produced from all recipient tobacco plants.

Light and electron microscopy

Light microscopic examination revealed amorphous inclusion bodies induced by PVY in epidermal strips cells of infected *N. tabacum* cv. White Burley 20 days post inoculation. They found to be granular, near the nucleus and stained in red **(Figure 2, A)**.

Electron microscopic examination of the same infected tobacco plants (30 days post inoculation) showed pinwheels, scrolls and laminated aggregates **(Figure 2, B)**.

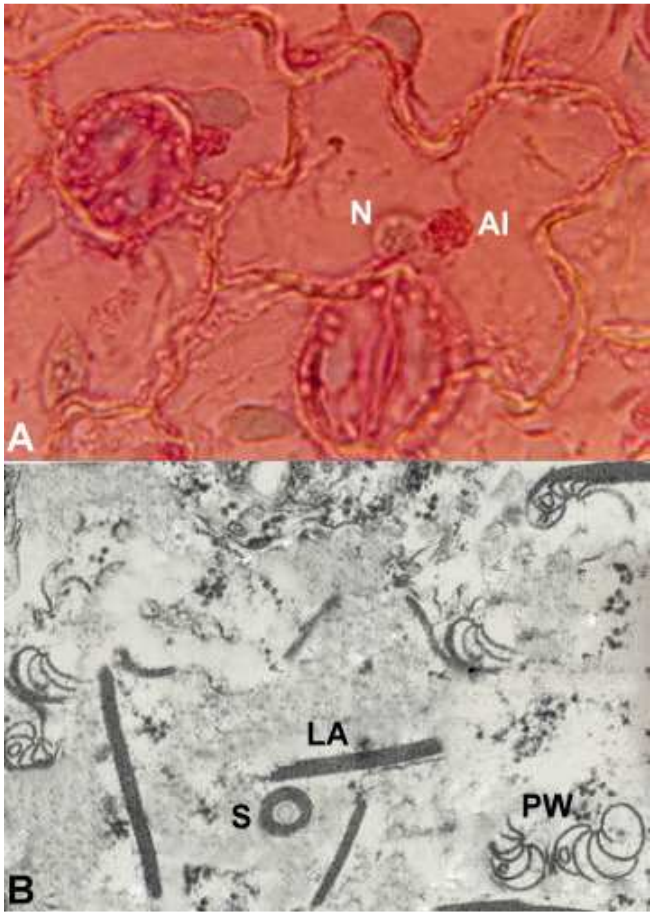

Figure (2): PVY red stained amorphous inclusions (AI) observed near the nucleus (N) in infected tobacco epidermal strips (A). Inclusion bodies induced by PVY in ultrathin sections of infected plants (B) in the form of pinwheels (PW), scrolls (S) and laminated aggregates (LA). Magnifications were 400× and 35000×, respectively.

Virus purification

Depending on UV absorption results of PVY purified preparation, virus concentration was calculated to be 3.1 mg/ml. The preparation appeared with good purity as A_{260}/A_{280} ratio was 1.51.

Electron micrograph of PVY purified preparation **(Figure 3)** showed flexuous filaments particles with model length of 730×13 nm.

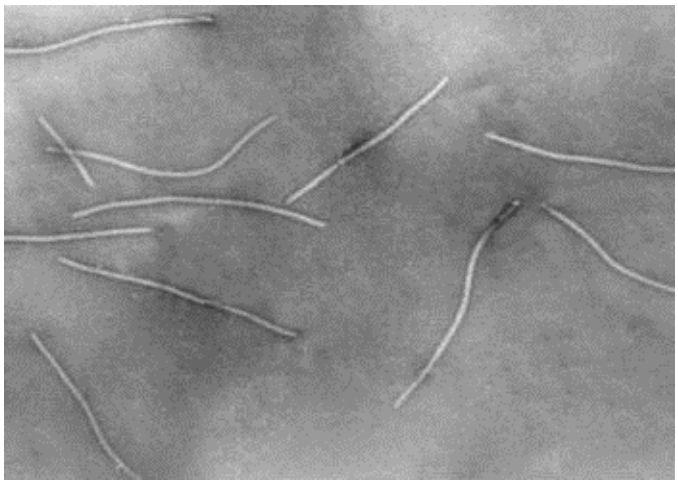

Figure (3): Electron micrographs of negatively stained purified PVY preparation, virus particles are flexuous filaments particles with model length of 730×13 nm, Magnification was 45000×.

Antiserum production and evaluation

For the PVY isolate, a specific antiserum was produced, IgGs were purified and its titer was determined using I-ELISA. Data in **Table (3)** show that antiserum reacted up to dilution of 1/1024 with clarified infectious sap while no positive results were obtained with the healthy tobacco sap.

Table (3): PVY IgGs evaluation against infected and healthy tobacco sap

Dilution		I-ELISA values at 405 nm*			
		Infected	R	Healthy	R
Crude		0.900	+	0.200	-
1:1		0.955	+	0.166	-
1:2		0.901	+	0.095	-
1:4		0.897	+	0.091	-
1:8		0.800	+	0.089	-
1:16		0.765	+	0.079	-
1:32		0.712	+	0.070	-
1:64		0.691	+	0.077	-
1:128		0.674	+	0.050	-
1:256		0.595	+	0.067	-
1:512		0.504	+	0.055	-
1:1024		0.419	+	0.052	-
1:2048		0.201	-	0.042	-
1:4096		0.193	-	0.049	-
Purified**	1	0.988		+	
	2	1.032		+	

* Each ELISA result (R) was the average of three readings.
** Reaction of crude purified virus with crude antiserum.

Evaluation of purified IgGs in detecting PVY

a) Diffusion tests in agar

Positive results in the form of precipitation zones were observed in Ouchterlony agar 24 h post incubation **(Figure 4, A)**. No positive result was observed between antisera and healthy tobacco sap. Precipitation zones in agar were also observed with the simple single diffusion test in agar surrounding the infected tobacco tissues **(Figure 4, B)**, while no precipitation was found with the healthy tobacco tissues.

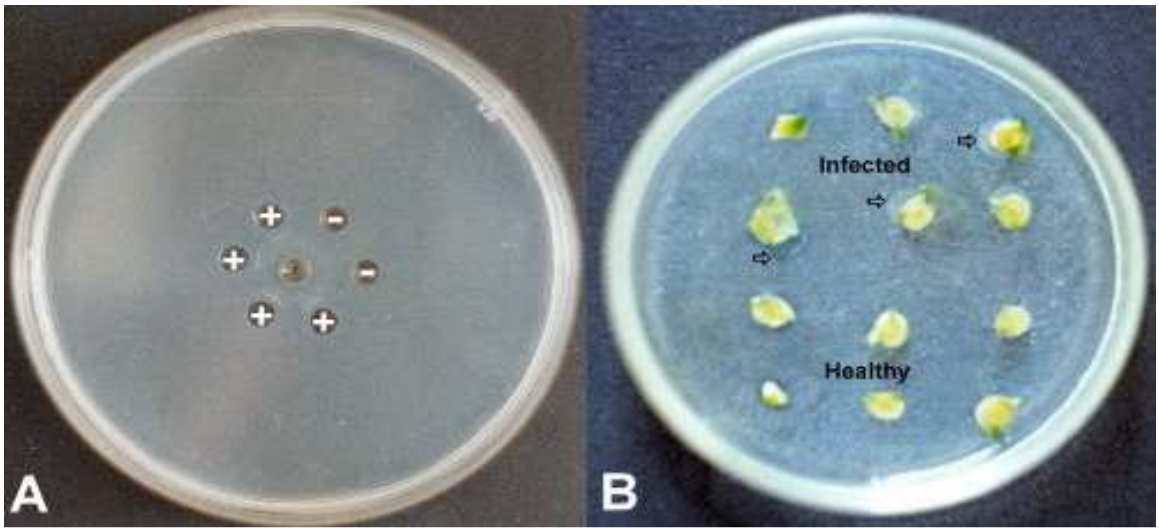

Figure (4): Double diffusion test in agar (A): positive results in the form of precipitation zones were observed with PVY infected sap, while no positive result was observed between antisera and healthy tobacco sap. A simple method for single diffusion test in plates (B): revealed zones of precipitation with infected tobacco tissues, while no reaction observed with healthy tissues.

b) Tissue and dot blot immunoassays (TBIA& DBIA)

TBIA & DBIA were used for IgGs evaluation by detecting the virus under study. TBIA gave positive results in the form of purple color development with the infected tobacco tissues, while no color was obtained with healthy samples **(Figure 5, A)**. Concerning DBIA, positive results in the form of clear purple color were obtained with the purified virus preparations and with the infectious sap dilutions till 10^{-6}, while no positive result was obtained with healthy tobacco sap **(Figure 5, B)**.

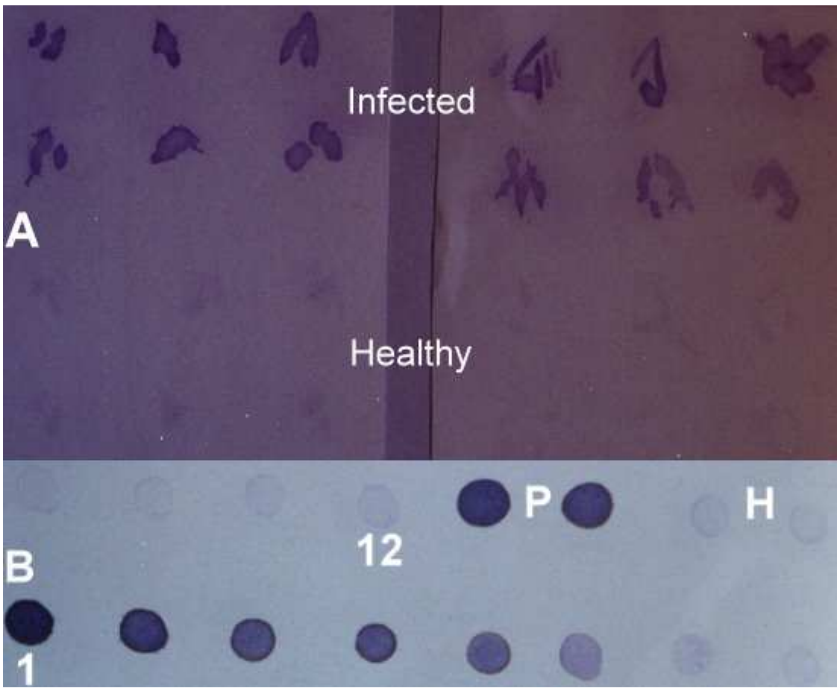

Figure (5): TBIA (A): positive reaction (purple color) was obtained with the PVY infected tissues, while negative result was found with control samples. DBIA (B): in a serial of tenfold dilutions of PVY infected tobacco sap (1-12), purified preparations (P) and the healthy sap (H). The last positively reacted point was 10^{-6}, purified preparation gave positive result while negative result was obtained with healthy sap.

SDS-PAGE of PVY coat protein

SDS-PAGE result illustrated in **Figure (6)** show that the molecular weights of PVY capsid protein was approximately 34 KDa, as bands with such size observed through acrylamide gel.

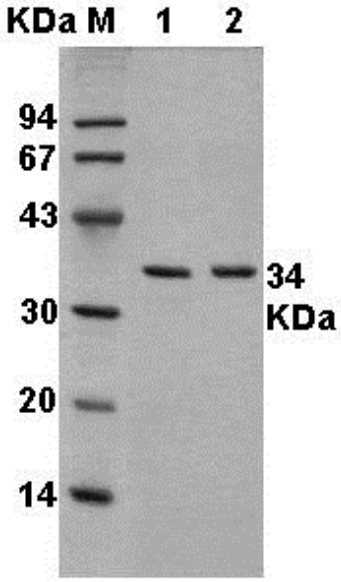

Figure (6): SDS-PAGE of purified PVY preparation (Lanes 1 and 2). M: Protein marker (Promega, USA).

Multiplex IC-RT-PCR for differentiation of PVY strain

Isolates representing three PVY groups: the understudy PVY strain, PVY^{O} and PVY^{N} (controls) isolates were tested. PCR agarose gel illustrated in **Figure (7)** show that all isolates tested gave products of the expected sizes as the understudy and PVY^{O} isolates gave products 267 and 689 bp, while PVY^{N} isolate produced PCR products of 328 and 398 bp.

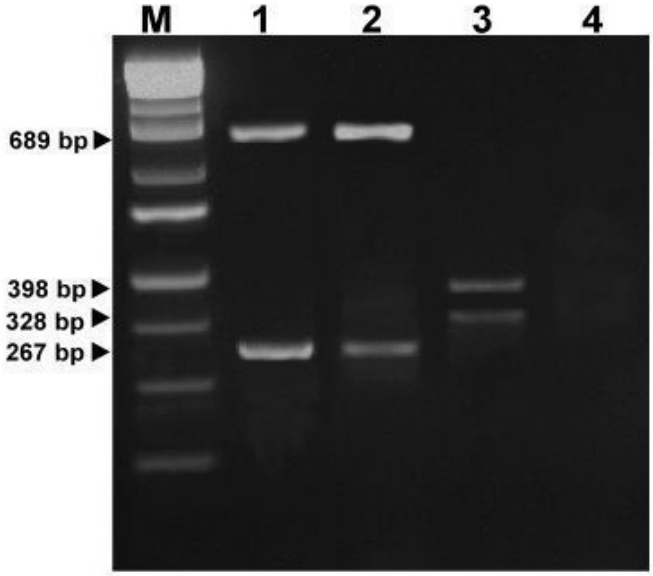

Figure (7): Multiplex IC-RT-PCR for the differentiation of the isolated PVY. Lanes 1, 2 representing tobacco tissue samples infected with understudy PVY and PVY^{O} isolates, respectively. While Lane 3, 4 were the product of PVY^{N} infected and healthy tobacco sap PCR, respectively. Lane M: DNA Ladder (Promega, USA).

DISCUSSION

Potato virus Y (PVY) is one of the most damaging plant viruses economically due to the importance of its plant host species, which include potato, pepper, tomato and tobacco. In connection with its wide host range, PVY displays a high variability and can be transmitted by insects in non-persistence manner **(Brunt, 2001; Kerlan, 2006)**. Potato strains are commonly subdivided into three main groups PVY^{O}, PVY^{C} and PVY^{N} **(De Bokx and Huttinga, 1981; Karasev and Gray, 2013)**; according to systemic and local symptoms, they induce on *Nicotinia tabacum*, *Physalis floridana* and *Solanum tuberosum*. However, in the last few decades, new strain variants have been reported, such as the tuber necrosis strain (PVY^{NTN}). Isolates of PVY^{NTN} are associated with potato tuber necrotic ringspot disease (PTNRD) **(Beczner et al., 1984; Le Romancer and Kerlan, 1991; Chikh-Ali et al., 2013)**.

All of PVY strains can be confirmed and identified by their abilities to induce inclusion bodies in infected tobacco cells, which can be detected as amorphous inclusions by light microscope and as cytoplasmic cylindrical inclusions that appeared under electron microscope in the form of pinwheels, scrolls and laminated aggregates **(Edwardson et al., 1984; Felczak et al., 2010)**.

Electron micrograph flexuous filaments particles for PVY with model length of 730×13 nm that was in accordance with (**Milne, 1988; Sabir, 2012)**.

The dilution end for the purified IgGs were determined using indirect ELISA. Data show that the highest titer of IgGs was 1:1024. These results were in harmony with those found by **Abdel-Salam et al. (1989)**.

Using agar diffusion tests PVY strains were detected and identified by many workers showing that positive reactions as a precipitation in agar **(Slack and Shepherd, 1975; Bodaghi et al., 2000; Ahmed, 2013)**.

Tissue blot used for IgGs evaluation by directly detecting the understudy virus in infected tobacco tissues. The test gave positive results in the form of purple color development. These results were in harmony with that found by **Koenig and Burgermeister (1986)** and **Asins et al. (2004)**. Dot blot immunobinding assay gave positive results in the form of clear purple color were obtained with the purified virus preparations and with the infectious sap dilutions till 10^{-6}. Similar trend was found with different plant viruses by **Powell (1987) and Kamenova and Adkins (2003)**.

Result show that the molecular weight of PVY capsid protein was approximately 34 KDa when estimated by SDS-PAGE. The results were in full agreement with those of **Yao et al. (1993)** and **Quintero-Ferrer Karasev (2013)**.

Use of Multiplex RT-PCR (with six pairs of specific primers) has shown that the understudy isolate belonging to the PVY^{O} strain which is currently the form of PVY infecting potato as it gave a two expected band sizes of 267 and 689 bp which is similar to reports from other researchers **(Lorenzen et al., 2006)**.

Lorenzen et al. (2006) report on a new multiplex assay that can identify each of the major PVY strains and strain mixtures in a single RT-PCR reaction. This assay was useful in identifying strain mixtures in two archived collections of PVY isolates. It should be useful for other researchers and seed certification agencies that would like to identify PVY strains and strain mixtures.

Chikh-Ali et al. (2006) used a multiplex RT-PCR assay which is previously developed by **Lorenzen et al. (2006)** to identify a group of PVY isolates with unusual recombinant structures (e.g., PVY^{NTN-NW} and SYR-III) and to differentiate them from other PVY strains. To make the multiplex RT-PCR assay more applicable and suitable for routine virus testing and typing, they modified the previous method by replacing the conventional RNA extraction step with the immunocapture (IC) procedure. The results obtained revealed that this multiplex RT-PCR assay is an accurate and robust method to identify and differentiate the nine PVY strains reported to date, including PVY^{O} (both PVY^{O} and PVY^{O}-O5), PVY^{N}, PVY^{NA-N}, PVY^{NTN}, PVY^{Z}, PVY^{E}, PVY-NE11, PVY^{N-Wi}, and $PVY^{N:O}$, which is not possible by any of the previously reported RT-PCR procedures. This would make the IC-RT-PCR procedure presented a method of choice to identify PVY strains and assess the strain composition of PVY in a given area. The IC-RT-PCR protocol was successfully applied for typing PVY isolates in potato leaf tissue collected in the fields.

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